AN INVESTIGATION OF NON-FLUORESCENT ANTINEUTROPHILS
AS POTENTIAL MEDIATORS OF FOCAL
MESANGIAL CELL LYSIS

JEFFREY SIEGEL BARKIN

1967

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AN INVESTIGATION OF NOVEL FLOURESCENT ANTIMETABOLITES AS POTENTIAL MOLECULAR PROBES IN NEOPLASTIC CELLS

A Thesis

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Department of Pharmacology

In partial fulfillment of the requirements for the degree of

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by

Jeffrey Samuel Barkin

1987

ABSTRACT

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Many 2,4-diamino-pteridines are potent inhibitors of dihydrofolate reductase; two novel trimethoxy-pteridines were synthesized. Flourescence characteristics of these two compounds were assessed, as were their ultraviolet and visible spectra. The first compound, 1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethylene) ('PKC-1'), was unable to inhibit dihydrofolate reductase (DHFR) derived from both a mammalian leukemic cell line and a bacterial source, Lactobacillus caseii. The compound 7,8-dihydro form ('PKC-2') was unable to inhibit either form of DHFR as well. As neither compound was an inhibitor of DHFR in vitro , it was not surprising that they demonstrated no appreciable cytotoxicity against a leukemic cell line (CEM) or a human colon carcinoma derived solid tumor. The concentration necessary to reduce the number of cells growing in culture by half was greater than 1 x 10^{-4} M. Both PKC-1 and PKC-2 are flourescent. A compound which is flourescent and avidly binds to and inhibits an enzyme such as DHFR would be very useful in elucidating the intracellular pathways of reduced folates. Further understanding the pathways of folates would aid in the development of new anti-folates, which themselves may circumvent some drug-resistance mechanisms in cancer cells.

5-flouro-2'-deoxyuridine-ethyl(7-methoxy)coumarin (FdUrd-MmC) is a strongly flourescent analog of deoxyuridine. After this compound was synthesized, its flourescence characterisitics were obtained, as were ultraviolet and visible spectra. FdUrd-MmC was unable to passively enter cells, as assessed by flourescence microscopy. It had no appreciable cytotoxic effect against either a leukemic cell line (CEM) or a solid tumor. FdUrd-MmC was forced to enter cells using electroporation, a technique which enables the introduction of otherwise impermeable compounds. Once introduced into CEM cells, FdUrd-MmC was not cytotoxic. As measured by a tritium release assay, FdUrd-Mmc transiently decreased DNA synthesis by ninety per-cent. Electroporation alone was also noted to decrease DNA synthesis. Flourescent nucleotide analogs may help elucidate molecular pathways of DNA synthesis by allowing direct visualization of cells under a flourescence microscope. This would be particularly useful in defining differences between neoplastic and normal cells.

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Dr. Pauline Chang synthesized FdUrd-MmC and the PKC 'series' of compounds, which bear her initials. Barbara A. Moroson guided me through the tissue culture lab and never hesitated to help me out when problems arose. Margaret Jastreboff, and Ramaswamy Narayanan patiently discussed new ideas and taught me the technique of electroporation. Dawne Newcombe helped with many references and always kept my spirits up. I am and honored to have worked with this group of people.

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Table of contents

	page
Introduction	1
Materials and Methods	19
Results	24
Discussion	39
Bibliography	43

LIST OF FIGURES

		Page
Figure 1:	Intracellular interconversions of folates.	4
Figure 2:	Structure of classical anti-folates.	5
Figure 3:	Mechanism of MTX cytotoxicity.	6
Figure 4:	Structure of non-classical anti-folates.	10
Figure 5:	Structures of PKC-1 and PKC-2.	14
Figure 6:	Structure of flourescein-labelled MTX.	15
Figure 7:	Structure of FdUrd-MmC.	16
Figure 8:	UV and visible spectra of PKC-1 and PKC-2.	25
Figure 9:	Flourescence spectra of PKC-1.	26
Figure 10:	UV and visible spectra of FdUrd-MmC.	30
Figure 11:	Mass spectroscopy of FdUrd-MmC.	31
Figure 12:	Flourescence spectra of FdUrd-MmC.	32

LIST OF TABLES

		Page
Table 1:	Structure-activity relationships of various 2,4-diamino quinazolines.	11
Table 2:	ED_{50} of various 2,4-diamino quinazolines in cell culture.	12
Table 3:	Tritium release from ^{3}H-2'-deoxyuridine (DNA synthesis activity).	40

ABBREVIATIONS

DHFR	Dihydrofolate Reductase
DMSO	Dimethyl Sulfoxide
FACS	Flourescence Activated Cell Sorting
FdUrd	5-flouro-2'-deoxyuridine
FdUrd-MmC	5-flouro-2'-deoxyuridine-ethyl(7-methoxy)coumarin
FH_2	dihydrofolate
FH_4	tetrahydrofolate
FUDR	5-flouro-uridine
HPLC	High Pressure Liquid Chromatography
MmC	4-bromoethyl-7-methoxycoumarin
MTX	Methotrexate
PBS	Phosphate Buffered Saline
PKC-1	1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxy-phenyl(ethylene)
PKC-2	1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxy-phenyl(ethane)
S.C.	Squamous Cell derived Solid Tumor
TMQ	Trimetrexate
Tris	tris(hydroxymethyl)aminomethane
TZT	Baker's antifolate

INTRODUCTION

Despite advances in cancer therapies, neoplastic disease still is the second leading cause of death in the United States, with approximately 5 million cases prevalent in the population in 1986 (Feldman, et al 1986). The three conventional modalities of cancer therapy, surgery, radiation therapy, and chemotherapy, have all been steadily advancing in efficacy. Surgery and radiation therapy are local therapies that aggressively destroy tumor at a specific site but do not impact on tumor metastases; rather, systemic therapy, such as chemotherapy, offers a method of reducing tumor burden insofar as the agents can reach the cancer cells, which themselves must be susceptible to the drugs. Combining modalities, such as the use of local surgical excision with adjuvant chemotherapy, have been described as reasonable alternatives to more radical surgery(Cummings, et al 1985). Unfortunately, current therapies are usually not curative, with the exception of a limited number of tumors (DeVita, 1985), such as embryonal carcinoma of the testis, acute lymphocytic leukemia, diffuse histiocytic lymphoma, Burkitt's lymphoma, certain childhood leukemias, and Hodgkins lymphoma. In other tumors therapy can offer temporary tumor regressions but in the majority of human cancers do not drastically impact upon overall survival rates.

Over the past twenty years, molecular biology has opened up new vistas into the functioning of cells and their genetic machinery. This basic knowledge has created deep insights into the molecular functioning of cells which in turn has allowed the development of drugs that disrupt various aspects of the cell cycle. Medical oncology strives to selectively upset the metabolism of the neoplastic cell while leaving the 'normal' cell intact. Therefore, cancer chemotherapy attempts to exploit differences between neoplastic cells and their normal counterparts in an effort to achieve a signinicant (usually several-log)kill. Limitations of chemotherapy include significant, potentially fatal, toxicity to the patient. Furthermore, many cancer cells develop resistance to chemotherapeutic agents, thereby rendering drug therapy ultimately ineffective. Some of these mechanisms of drug resistance will be discussed later in this paper. First , an overview of one chemotherapeutic mechanism will be presented. The relationships of drug resistance to chemotherapeutic maneuvers and specifically ways of bypassing a mechanism of drug resistance in order to obtain a novel anti-cancer drug will be discussed later in this paper.

1. Folate depletion,'classical' anti-folates and cancer chemotherapy:

In 1947, dramatic remissions were obtained in childhood leukemias after treatment with the folic acid analog, aminopterin(Farber, et al 1947). The hypothesis that folate depletion was responsible for the antineoplastic effects of aminopterin prompted detailed investigation into other folate depleting strategies. It was noted that many of the compounds which deplete intracellular folate are potent inhibitors of the enzyme dihydrofolate reductase (DHFR). DHFR inhibition results in a global depression of other enzymes which utilize folate as a co-factor. Figure 1 illustrates the relationships of folates to some of its' varied pathways. The folate co-enzymes participate in many diverse important reactions such as the synthesis of purines, thymidylate, as well as the amino acids methionine and serine. Thus, inhibition of DHFR by an anti-folate results in significant decreases of the purines and thymidylate, which are necessary for DNA synthesis. The nucleotide pool needed by rapidly replicating neoplastic cells is greater than normal cells. Hence, drugs which decrease the nucleotide pool suppress the growth of these disordered neoplastic cells.

'Classical' anti-folates, typified by methotrexate(MTX), have a 2,4-diamino-structure and a glutamic acid moiety. Figure 2 shows the structures of folic acid as well as the 'classical' anti-folates, methotrexate (the most widely employed anti-folate in use clinically) and aminopterin, which is identical to folic acid except that it has an amino group on the 4 position as opposed to a hydroxyl group. Methotrexate is similar to aminopterin except that it has the addition of a 10-methyl group (Jackson, 1984; Baker, 1976). MTX competes with dihydrofolate reversibly, but extremely effectively, with IC_{50} values in the range of 1×10^{-9}M at pH 7.0 (Bertino, et al 1964). Once inside the cell, MTX can become polyglutamated, and not lose its activity against DHFR (Rosenblatt, et al 1978). Figure 3 graphically depicts the sites of MTX activity. Furthermore, MTX is actively transported into cells in such a way that very high dose MTX therapy competitively blocks the entry of other folates directly into cells. This advantage has been exploited clinically: Patients receive very high doses (grams/m^2) of MTX to obtain the highest possible intracellular concentration, and are then "rescued" with N^{10}-formyltetrahydrofolate (leucovorin)(Sirotnak, et al 1974). That MTX requires an active uptake mechanism also allows cells a mechanism to become resistant to this drug. Before discussing the so called 'non-classical' anti-folates, a discussion of drug resistance will be offered. Various ways of circumventing some of the mechanisms of drug resistance and how this specifically applies to the design of novel anti-folates will be described.

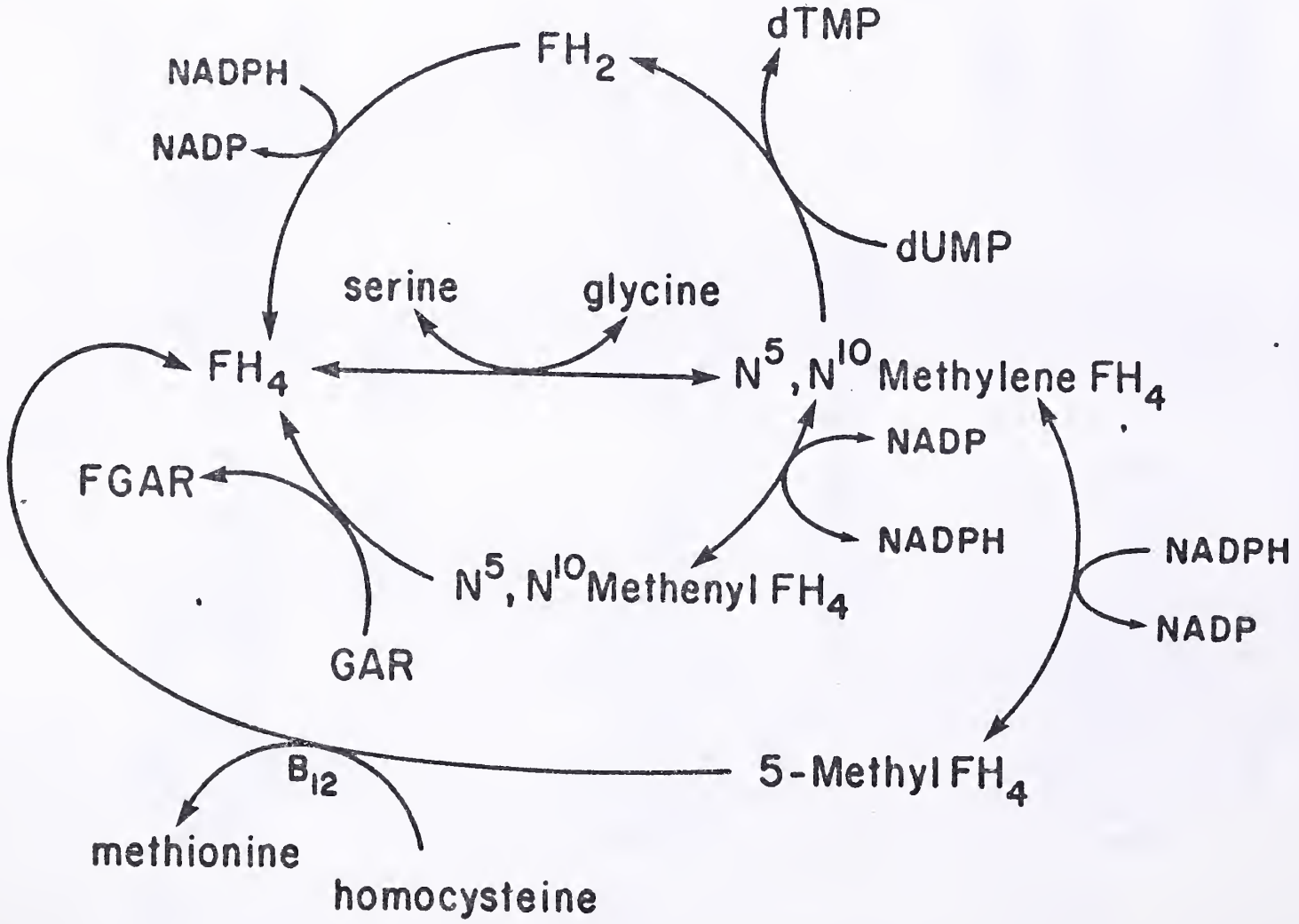
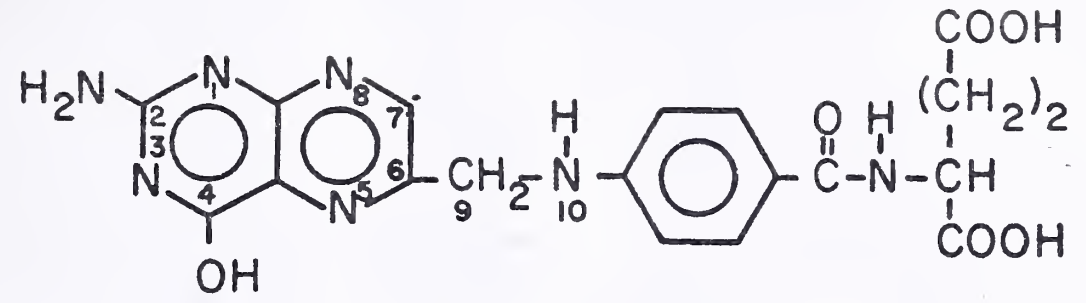
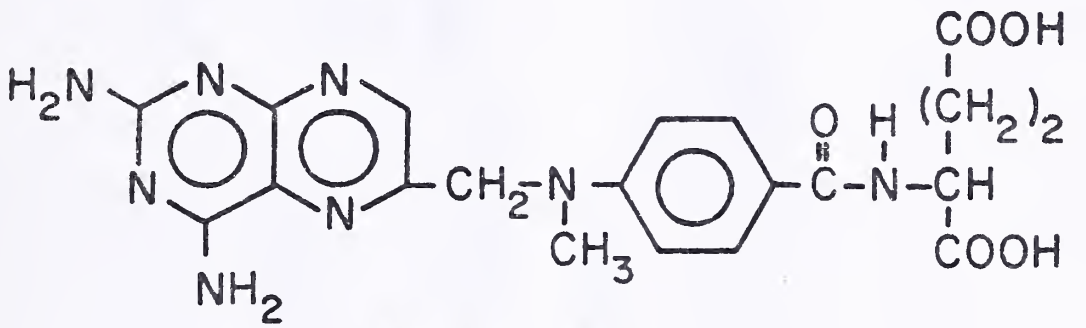


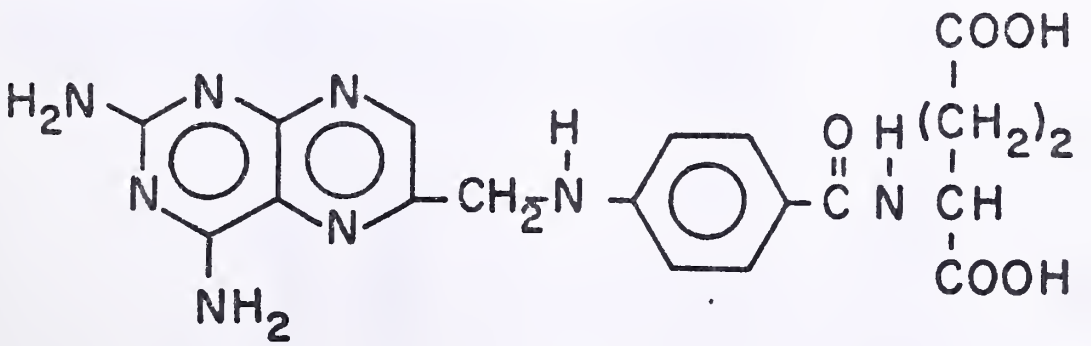
Figure 1.
Important interconversions of folates. DHFR converts FH_2 to FH_4. Thymidylate synthetase converts dUMP to dTMP.



FOLIC ACID



METHOTREXATE



AMINOPTERIN

Figure 2.
Structure of folic acid and the classical folic acid antagonists, methotrexate and aminopterin

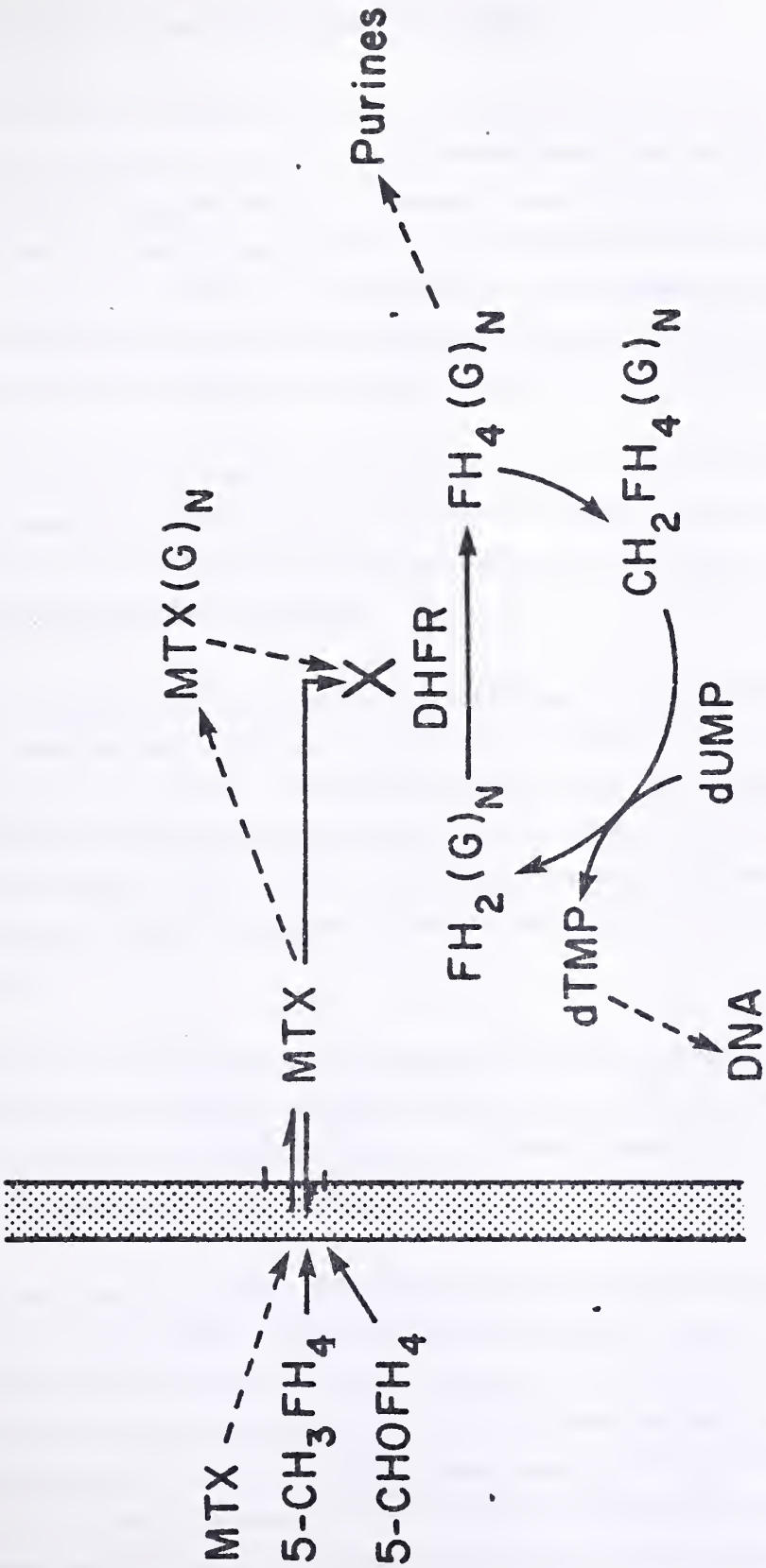


Figure 3.
Diagram of MTX actively entering cells, inhibiting DHFR and becomming poly-glutamated.

2. Mechanisms of drug resistance to chemotherapeutic agents:

As previously stated, chemotherapy produces varying degrees of clinical responses in many cancers. Frequently, relapses occur and the tumor is often drug resistant making a second remission difficult to achieve. Therefore, drug resistance imposes a major limitation upon the treatment of many human cancers. Resistance may be broadly broken down into two types. Firstly, inherent resistance of a tumor, melanoma, and non-small cell lung cancer being prime examples, imply poor susceptibility to chemotherapeutics and, not surprisingly, poor clinical responses (DeVita et al, 1985). Secondly, acquired drug-resistance implies patients whose tumors have relapsed after drug treatment. There are a very large number of mechanisms of acquired drug resistance, some of which will be described in this paper.

Many mechanisms of drug resistance have been identified. Some of these mechanisms will next be explored, using MTX as a model drug. It must be recognized that cells may express a variety of these "escape mechanisms" at a given time, for if a cell developed an isolated change, such as increasing metabolic inactivation, than raising the dose of the drug may overcome the drug resistance.

Specific alterations in the target enzyme (DHFR) of MTX have been reported (Simonsen, et al 1983; Flintoff, et al 1980). A single nucleotide alteration in the gene coding for DHFR was noted to cause a marked reduction in the affinity of MTX for this enzyme. Indeed, there are several examples of this phenomena in different cell lines where altered DHFR's have been isolated and the binding of MTX to the enzyme have been radically changed (Bertino, et al 1985). In one murine cell line, L5178Y, a 100,000 fold decrease in MTX binding to a mutant DHFR has been noted(Goldie, et al 1980). Thus, nucleotide alterations in a target enzyme have delineated one mechanism of acquired drug resistance.

Treatment of cells with MTX led to the finding of yet another mechanism of drug resistance: An increase in intracellular DHFR levels (Alt, et al 1978). Not only is the level of DHFR increased but the DNA sequences that code for this enzyme become amplified. Bertino and co-workers (Bertino, et al 1983) describe murine, hamster and human MTX-resistant cells which were found to contain increased concentrations of DHFR.

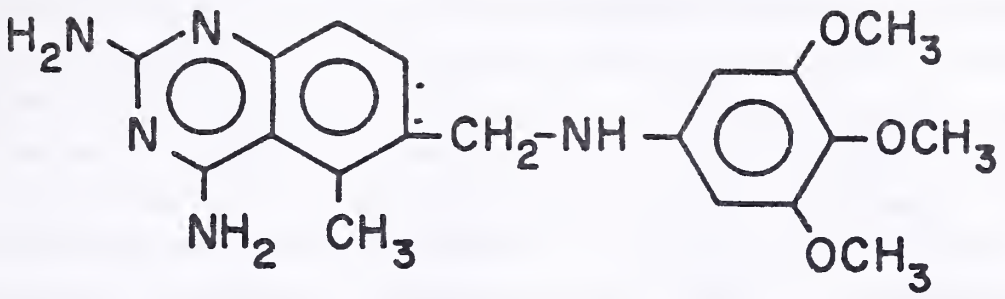
Additionally, analysis with cDNA probes disclosed amplified amounts of the gene which code for DHFR, and, not surprisingly, abundant mRNA which also codes for DHFR. When human cell lines are exposed to increasing concentrations of MTX, and the surviving clones are selected out in a serial fashion with increasing concentrations of MTX at each passage, at high levels of drug resistance homogenous staining regions are visible upon examination of their chromosomes(Srimatkanada, et al 1983). These changes in both chromosomal architecture and resistance to MTX are stable, and are demonstrable after six months of serial growth in MTX-free growth media. *In situ* hybridization studies conclusively demonstrated that the homogenous staining regions indeed were additional copies of the DHFR gene (Nunberg, et al 1978).

Another genetic pattern, which is unstable and therefore does not persist after treatment of cells previously made MTX resistant by serial culturing, demonstrates so-called "double minute" chromosomes, which are small generally paired extra-chromosomal clusters devoid of a centromere(Kaufman, et al 1979). After it became generally excepted that gene amplification is a mechanism of drug resistance in the MTX/DHFR model, it quickly became demonstrated that this mechanism operates in a wide variety of other drug/enzyme systems. For example, the same phenomenon has been illustrated for FUDR and the enzyme thymidylate synthetase (Priest, et al 1980).

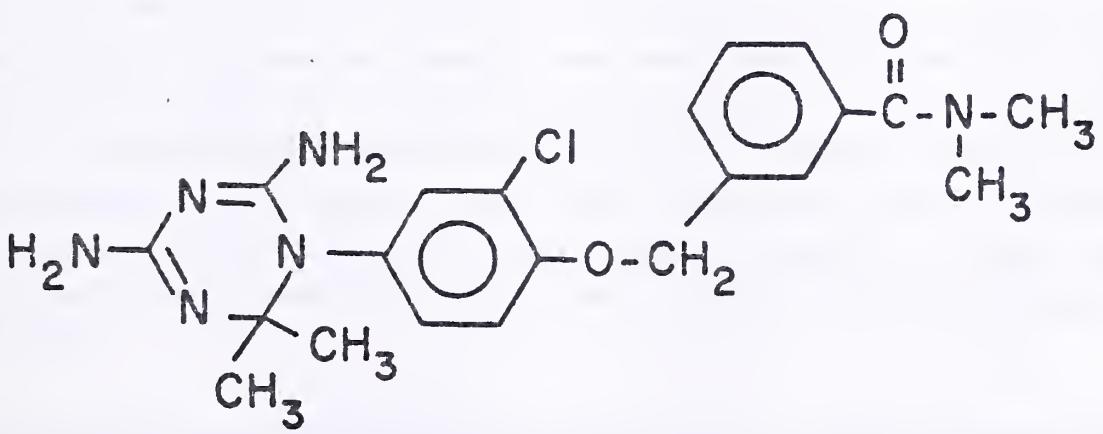
As stated previously, MTX relies upon an active transport system in order to obtain entry into cells. Sirotnak (Sirotnak, et al 1981) has demonstrated that defects in transport in murine *in vivo* systems were as common as elevated DHFR concentrations in causing MTX resistance. Since the loss of active uptake is a cause for drug resistance, at least in the MTX/DHFR system, it seems logical to attempt to devise new compounds which are potent DHFR inhibitors but do not rely upon active uptake to gain cellular entry. Alternatively, one can attempt to use other mechanisms, such as liposomes containing a drug that does not by itself gain entry (such as MTX) in an effort to overcome this type of resistance (Kaye, et al 1981).

A group of compounds, designated as 'non-classical' anti-folates have been synthesized which do not rely upon the active transport system to gain entry into cells. Some of these compounds have potent inhibitory effects on DHFR, and therefore represent an exciting new avenue to pursue in order to purposefully manufacture compounds which are immune to one of the three established mechanisms of drug resistance for anti-folates. These 2,4-diamino heterocycles lack the glutamate moiety of MTX and their structures will be discussed shortly.

The ultimate goal, then, is to obtain compounds which avoid the need for active uptake but that exhibit similar binding kinetics to DHFR like MTX. In order to protect normal cells, which have a functional active folate uptake system, pre-treatment with leucovorin (which requires an intact active uptake system) would seem logical. The neoplastic cells which have lost the active uptake system as a means of earlier drug resistance, would not take up the leucovorin and would therefore be preferentially susceptible to the toxic effects of the new drug. A hypothetical combination treatment plan logically follows: treat the tumor initially with MTX, obtaining a significant cell kill, and then test to see if remaining cells (if any) have acquired a transport defect. If they have, re-treat the patient with the new 'non-classical' anti-folate with concurrent leucovorin rescue of the non-cancerous cells(Bertino, 1984). In fact, many of these compounds have been synthesized and tested, demonstrating DHFR inhibition and an ability to passively diffuse through cell membranes, thereby successfully bypassing the active folate uptake system. Figure 4 demonstrates two of these compounds, TZT(Baker's antifolate) and trimetrexate(TMQ, JB-11). The latter drug is currently undergoing phase two clinical trials. What makes these novel compounds active? Can other compounds be made that share these unique properties? In this thesis two compounds,which are similar structurally to TMQ, are tested for their activity against cells in culture and against DHFR *in vitro*. These compounds were also analyzed in terms of their flourescence, ultraviolet and visible spectra. First, previously determined structure-activity relationships for this class of drugs will be reviewed, and attempts to make some generalizations about which compounds would most likely be suitable to manufacture and study will be offered. The ultimate goal of this research is to discover and characterize less toxic and more potent anti-neoplastic drugs, and in this case, compounds which bypass a well characterized mechanism of drug resistance.



TRIMETREXATE
(NSC 249008, JB-II, TMQ)



TZT
(NSC 139105, Baker's Antifol)

Figure 4.
Structure of the non-classical folic acid antagonists, Trimetrexate and TZT.

3. An overview of Structure-activity relationships of 'non-classical' anti-folates:

Bertino and his colleagues (Bertino, et al 1979) analyzed seventeen compounds as potential DHFR inhibitors. Table one presents the results of these studies and discloses the IC_{50} of the test compounds in two cell lines, a human acute lymphocytic leukemia and a MTX resistant murine leukemia(L 1210). All of these 2,4 -diaminoquinazolines were predicted to have some inhibitory effect on DHFR; further, these componds were designed to contain lipophilic regions which would confer a proclivity for passive diffusion across biological membranes. Alkyl, acyl,or -*NO*, groups were placed at regions corresponding to transfer groups in the tetrahydrofolate coenzymes. As shown in table one all of these compounds were potent inhibitors of DHFR. Compounds three, eleven and fourteen demonstrated appreciable activity in inhibiting both the human and murine derived DHFR's. The compounds were next tested in tissue culture (see table two) against the murine derived L1210 cell line. Compounds eleven and fourteen demonstrated excellent activity with an ED_{50} of roughly 1×10^{-8}M. The table further shows a strong relationship between inhibition of cell growth and inhibition of DHFR. Compound fourteen, (2,4-diamino-5-methyl-6-[3,4,5-trimethoxyanilino)-methyl]quinazoline,is trimetrexate (TMQ, NSC 249008),and is currently undergoing phase two clinical studies in humans, as work in other animal *in vivo* systems has demonstrated activity against several tumors. It has been further demonstrated that TMQ achieves high intracelluar concentrations in a MTX transport-resistant cell line, CCRF-CEM (R3) (Bertino, et al 1985; Mini, et al 1985). TMQ like MTX, when tested against a 3T6 cell line with normal transport properties, but which is resistant to MTX because of a mutant DHFR, also demonstrated a decreased affinity for this enzyme.

The work of Sirotnak and his group (Sirotnak, 1979) on structure-activity relationships of anti-folates yielded some interesting findings. For a compound that requires active carrier mediated uptake at least two conditions must be met in order for it to be cytotoxic. First, it must gain cellular entry and, second, it must bind to some active site. Referring to the structures in figure 4, Sirotnak demonstrated in L1210 cells(a murine leukemia line) that modifications at positions 1, 3, 4, 5 and 8 affect binding to DHFR, whereas position 10 does not. Furthermore, alterations at positions 4,5,8, and 10, but not positions 1 and 3, affect influx through the active transport system. Therefore, structural alterations at position 10 seem to uniquely affect cellular entry without modifying binding to the active site of DHFR.

From the data presented earlier for TMQ and the preceeding discussion, it is clear that it is possible to make enzyme inhibitors that do not rely upon the active transport system, and therefore obtain high intracellular concentrations in cell lines which no longer express the transport mechanism. The trimethoxy structure probably affords the compound sufficient lipophilicity to passively diffuse through biological membranes. Can a 10-deaza compound (one that has a carbon replacing the 10 nitrogen) maintain activity as an inhibitor of DHFR? Would such a compound exhibit flourescence? A flourescent compound which does not rely upon an active uptake system and avidly binds to DHFR would be extremely useful in demonstrating whether or not a specific tumor has lost the active uptake system.

Table one:

Structure-activity relationships of various 2,4-diamino-6[(anilino)-methyl] quinazolines to inhibit Dihydrofolate reductase derived from a murine (L1210) and a human (acute lymphocytic leukemia).

Reproduced from:

Bertino, J.R., Sawicki, B.A., Moroson, B.A., Cashmore, A.C., Elslager,E.F. 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline (TMQ). A potent non-classical folate antagonist inhibitor. Biochemical pharmacolgy 28: 1983-1987(1979)

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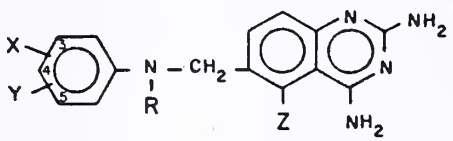

Compound No.*	NSC No.	X, Y	R	Z	ID_{50} (M × 10^9) ALL	L1210R
1	250412	3,4-Cl_2	H	H	3.8	9
2	250413	3,4-Cl_2	H	CH_3	2.6	2.9
3	208652	3,4-Cl_2	H	Cl	1.1	2.0
4	250414	3,4-Cl_2	NO	H	10	10
5	250415	3,4-Cl_2	CH_3	H	5	
6	250416	3,4-Cl_2	$COCH_3$	H	50	10
7	250417	3,4-Cl_2	NO	CH_3	10	
8	250657	3-Cl	H	H	10	10
9	250418	4-Cl	H	H	6	5
10	250419	3-Br	H	H	2.2	2.3
11	250420	3-Br	H	CH_3	1.5	1.5
12	250421	4-Cl, 3-CF_3	H	H	4	
13	250422	3,4,5-$(OCH_3)_3$	H	H	6	
14	249008	3,4,5-$(OCH_3)_3$	H	CH_3	1.3	1.2
15		2-Cl, 4-CH_3	CH_3	H	60	
16		3-Br	H	Cl	4.0	
17		4-F	CH_3	H	30	
Methotrexate					0.9	0.9

* All compounds were tested as the acetate salts with the exception of compounds 5–7 which were tested as the free base.

Compound	IC_{50} for DHFR from L1210 ($M \times 10^{-9}$)	ED_{50} for L1210(cells in culture) ($M \times 10^{-8}$)
14	1.2	0.7
11	1.5	1.0
9	5.0	2.5
1	9.0	4.0
8	10.0	4.8
6	10.0	6.0
10	2.3	6.0
4	10.0	9.0
MTX	0.9	0.8

Table two:

Activity of 2,4-diamino-6-[(anilino)methyl]quinazolines against L1210 cells grown *in vitro*

Reproduced from:

Bertino, J.R., Sawicki B.A., Moroson, B.A., Cashmore, A.C., Elslager, E.F. 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline (TMQ), A Potent Non-Classical Folate Antagonist Inhibitor. Biochemical Pharmacology 28: 1983-1987 (1979)

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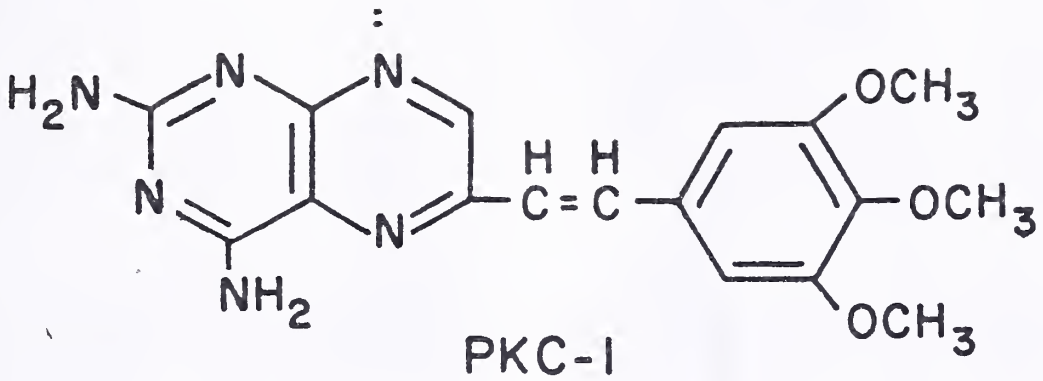
Thus, this analog would be of value in visualizing directly or with the aid of flourescence activated cell sorting (FACS) whether or not a cell line, which is resistant to 'classical' anti-folates, has acquired a transport defect. Figure 5 demonstrates two such compounds with such a carbon for nitrogen substitution. The remainder of the structure is very similar to TMQ, sharing the trimethoxy-benzene lipophilic end-region, although both of these new compounds are pteridine and not qunazoline analogs. The top compound, 1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethylene) ("PKC-1") was first synthesized; reduction of the carbon-carbon double bond yielded the second structure, identified as the 7,8-dihydro structure, 1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethane) ("PKC-2"), as depicted. These compounds were then tested for their ability to inhibit DHFR in an enzyme assay; they were also tested for their ability to inhibit the growth of human cancer cells in tissue culture. This data will be presented later, as will the flourescence, ultraviolet and visible spectra of these two new compounds.

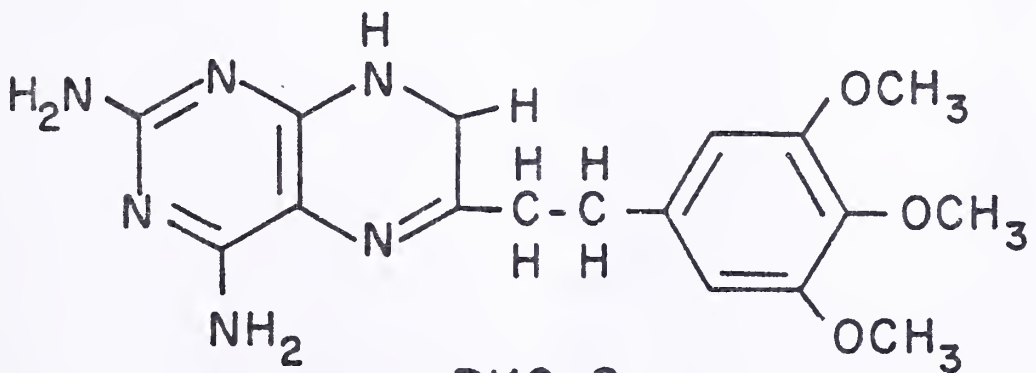
4. Flourescent analogs useful in the measurement of intracellular enzymes:

Figure 6 demonstrates the structure of a flourescein labelled MTX which allowed measuring the variations of DHFR levels intracellularly, using a flourescence activated cell sorter(FACS) (Gapski, et al 1975). This technique allowed measurements of DHFR within a cell line and confirmed that after exposure to MTX elevations in DHFR concentration occur; after serial generations in a MTX-free media, DHFR levels decreased(Bertino, 1978). Similarly, flourodeoxyuridine (FdUrd) was made flourescent by conjugation with 4-bromomethyl-7-methoxycoumarin(MmC), yielding FdUrd-MmC (figure 7). The flourecsent and mass spectroscopic spectra of this compound were determined and will be presented. The goal in the synthesis of this compound was to determine if it entered cells, as measured by intracellular flourescence. If it did enter cells, was it capable of inhibiting DNA synthesis, and was it able to inhibit cell growth? ^{3}H release after the addition of ^{3}H-deoxyuridine was used to measure both DNA synthesis and thymidylate synthase activity. If this flourescent compound is incorporated into DNA, it may be possible to observe aspects of the cell cycle directly via flourescence microscopy. The ability of this compound to inhibit cell growth, enter cells passively, and inhibit DNA synthesis was measured and will be reported.

This flourescent analog was also useful in demonstrating the effectiveness of electroporation, a relatively new technique that allows compounds that do not usually enter cells to be efficiently introduced(Knight, et al 1986). Electroporation consists of a brief, high-voltage electric discharge which apparently creates transient pores in the plasma membrane, allowing the introduction of usually impermeable compounds. Recently, this technique has been used to introduce deoxyribonucleoside triphosphates into intact cells (Sokoloski, et al 1986). The technique has also been shown to introduce 5-flouro-2'-deoxyuridine 5'-monophosphate into intact cells. This compund is an active thymidylate synthetase inhibitor, and therefore DNA synthesis inhibitor.



PKC-1



PKC-2

Figure 5.
Structure of PKC-1 (1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethylene).
and PKC-2 (1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethane).

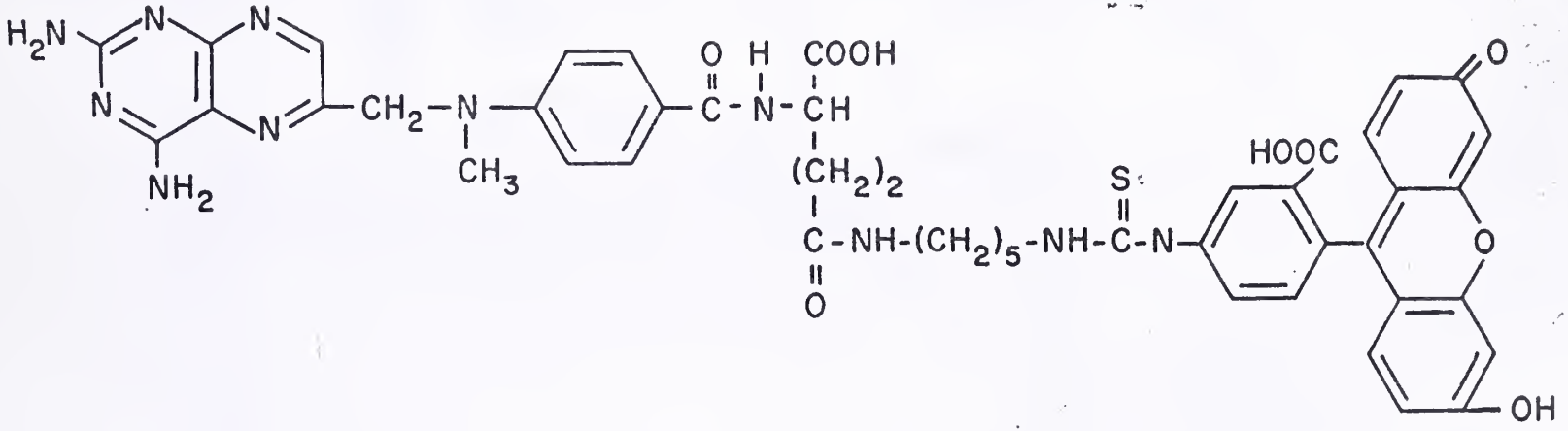
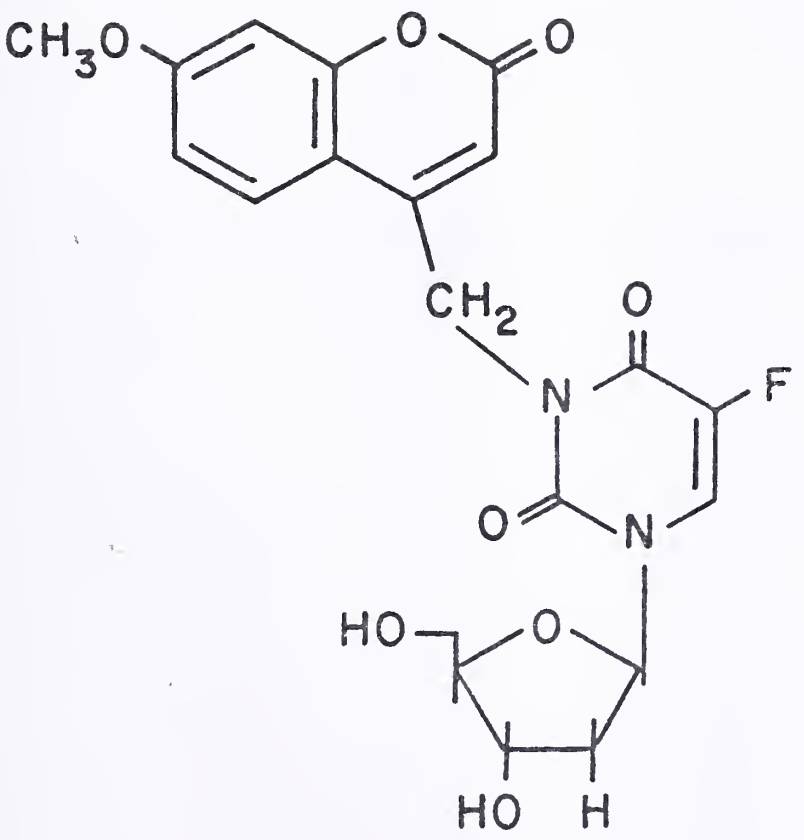


Figure 6.
Structure of Flourescein-labelled Methotrexate.



3-Mmc-FdUrd

Figure 7.
Structure of 5-flouro-2'-deoxyuridine conjugated with 7-methoxycoumarin (FdUrd-MmC).

Thus, electroporation has allowed the introduction of an impermeable cytotoxic compound directly into cells(Jastreboff, et al in press). The flourescent analog of FdUrd would be a prime candidate to quantitatively study the effectiveness of electroporation by ascertaining what percentage of cells become flourescent. Finally, electroporation was used to introduce FdUrd-MmC into a human leukemic cell line with subsequent measuring of these cells viability and their capacity to synthesize DNA. These preliminary data will be presented as will be suggestions for further research.

MATERIALS AND METHODS

1. Spectroscopy:

Ultraviolet and visible frequency spectra were obtained by making a stock solution of test compounds, PKC-1 (double bond compound), and PKC-2 (the 7,8-dihydro form), containing 12.7 and 3 milligrams respectively dissolved in 1 ml DMSO. 20 ul of the stock solutions were then added to the indicated solvents, which consisted of HPLC grade methanol, 0.05 M K_2PO_4 which was adjusted to pH 7.0, 0.01 M HCl, and 0.1 M NaOH. The total volume in the cuvette was adjusted to 1.00 ml in all cases. An identical procedure was performed in the case of FdUrd-MmC, which utilized HPLC grade methanol as the solvent. A Cary 15 model spectrophotometer was employed to measure all UV and visible spectra; the molar absorption maximum was determined by finding the peak absorption within the slit width of the device. Spectra were repeated the following day to determine stability of the test compounds.

Flourescence spectra were obtained using a Foci brand Farrand Optical Spectroflourometer model 801. Emission and excitation frequencies were determined; once the excitation frequency was obtained that produced maximum flourescence another scan was performed to determine the emission spectra of the test compound, holding the excitation frequency constant. Spectra were determined in the solvents as shown in the results section and were chosen to represent a range of pH's and ionic characteristics. Flourescence spectra were repeated the following day to determine if the compounds maintained their flourescence characteristics.

2. Dihydrofolate Reductase Enzyme Assay:

L5178Y derived DHFR was employed to test compounds as potential inhibitors of this enzyme. The reaction mixture occured in a total volume of 1.0 ml in each cuvette (Gupta, et al 1977). Final concentrations of reagants in this reaction yielded 100 uM Tris-HCl buffer, pH 7.0; 150 uM KCl; 0.08 uM NADPH; and albumin 1 mg/ml. To this mix, 0.55 ml of H_2O was added; if an enzyme inhibitor was to be tested, it was added in a volume of 0.10 ml and 0.45 ml H_2O was added to obtain a constant volume. Initially, the enzyme inhibitors were dissolved in 1 ml of DMSO, and then diluted in H_2O to obtain the appropriate final concentration in the reaction mixture. The concentration of enzyme was adjusted in the abscence of inhibitors so as to yield a change in optical density of 0.020/minute at 37 C. The amount of enzyme was therefore controlled for each reaction and held constant. This mixture was then incubated at 37 C for two minutes in the prescence of 50 ul of DHFR extract. Subsequently, 0.02 uM of FH_2 was added, which contained 10 uM 2-mercaptoethanol, in a total volume of 50 ul. FH_2 was prepared by dissolution in 0.5 M Tris HCl, pH 8.5 and adjusted with H_2O for a final concentration of 1 um/ml. Activity as um/hr/ml was calculated by measuring the change in optical density at 340 nm over a five minute period. Activity in um/hr/ml therefore =

$$\frac{\text{change in optical denisty at 340 nm}}{\text{5 minutes}} \times \frac{\text{60 minutes}}{\text{hour}} \times \frac{1}{\text{absorbance coefficient}} \times \frac{1}{.05 \text{ ml enzyme extract}}$$

Spectrophotometric measuring of optical density employed a Gilford Model 2000 recorder. The change in optical density reflects the dissapearance of NADPH as described by the reaction below:

$$FH_2 + NADPH \xrightarrow{DHFR} FH_4 + NADP$$

3. Cell Culture Procedures:

CCRF-CEM(Foley, et al 1965) leukemia cells and SC squamous cell carcinoma lines growing exponentially in RPMI with 10 % fetal horse serum (RPMI 1640 + 10 % fetal horse serum ="medium") were then diluted into fresh media for a concentration of 5×10^4 cells/ ml. Cell counts were accomplished using a Coulter counter. Fresh cells were distributed into a final volume of 10 ml in cell culture tubes; test compounds were added to these culture tubes so as to achieve various concentrations of inhibitor (added volume of 0.1 ml of inhibitor to culture tubes). All concentrations, as well as controls which were free of inhibitor (test compound) were run in duplicate.

CEM-CCRF cells incubated at 37 C as a suspension for forty-eight hours were then counted directly with a Coulter counter. If the cell suspension was very concentrated, it was diluted by a factor of ten in normal saline and then counted. An identical procedure was followed for the SC cells, except they grow as a monolayer. They were therefore removed from the culture tubes by a twenty minute incubation with trypsin, ticturated, and finally re-suspended in fresh growth media prior to counting.

The effective dose 50 % (ED_{50}) was extrapolated as the concentration of inhibitor required to decrease the cell number to fifty per cent the control value (no inhibitor added) after an incubation period of forty-eight hours. This was derived by plotting the cell number derived from the Coulter counter against the dose of the inhibitor employed. As all concentrations were done in duplicate, average values were used for the above calculation.

As these experiments were done more than once and the cell lines were needed for many trials, stocks of cells were carefully maintained and checked twice per week for possible bacterial contamination. There were no signs of contamination at any point throughout the duration of this work. Furthermore, the abscence of mycoplasma infections was periodically documented by the director of the tissue culture facility.

4. Thymidylate synthetase assay:

Deoxyuridine monophosphate is converted to deoxythymidine monophosphate by the enzyme thymidylate synthetase; in the process the 5-hydrogen detaches from the uridine. After uptake by cells of [5-^{3}H]-2'-deoxyuridine and its subsequent phosphorylation to [5-^{3}H]dUMP, the tritium is released as $^{3}H_2O$ into the growth media. A methyl group replaces this hydrogen, thereby forming thymidine monophosphate. Cells were diluted in medium (RPMI + 10 % fetal horse serum) to a concentration of 4 x 10 ^{5} cells per ml(Yalowich, et al 1985). The cells were then divided into 400 ul aliquots and placed in 12 x 75 mm culture tubes. [5-^{3}H]-2'-deoxyuridine was added so as to obtain a final concentration of 1 uCi per ml; 100 ul of this mixture was taken at 0, 15, 30 and 45 minutes and added to 200 ul of 15 % activated charcoal suspension in 3 % trichloroacetic acid. The mixture was then centrifuged at 16,000 x g for 5 minutes; 100 ul of the supernatant was then added to 5 ml of scintillation fluid and counted.

The above procedure was performed after electroporation (described below) of FdUrd-MmC into cells. Four groups of cells were tested: 1)no compound and no electroporation (control); 2)compound was added but not electroporated; 3)cells were electroporated but no compound was added and ; 4)compound was both added and electroporated. (The specifics of this procedure will be discussed in the section describing electroporation.) These four groups were assayed at four time points after the electroporation: 2 hours, 3 hours, 4 hours and twenty-four hours. Scintillation counts were analyzed using linear regression to calculate the slopes of the best linear ^{3}H release curves; data are expressed as the percentage of the slope of the control cells (neither electroporated nor drug added)(Rodenhuis, et al 1986).

5. Electroporation of FdUrd-MmC:

FdUrd-MmC was dissolved in 0.5ml of 50% HPLC grade methanol and 50 % phosphate buffered saline (PBS) to achieve a concentration of 20 mM. Initially, the FdUrd-MmC dissolved without difficulty, but on the morning of th experiment some precipitation was noted. Attempts to warm and heat the solution afforded further dissolution but some crystals remained undissolved. This solution of FdUrd-MmC was employed, as addition of 100 % pure methanol would have injured the cells.

Techniques for electrporation of the compound were followed as described by Jastreboff(Jastreboff, et al in press). Exponentially growing CEM-CCRF cells were washed with PBS and then collected by centrifugation (all centrifugations in this section were 1100 RPM for six minutes at 37 C) into a pellet. The pellet was then resuspended in PBS to achieve a cell concentration of 1.5×10^7 cells per ml. The cells were then cooled on ice for ten minutes after being divided into four small tubes, each containing 0.5 ml (approximately 1×10^7 cells). Two of these tubes received 50 ul of the stock solution containing FdUrd-MmC (for a final concentration of approximately 1 mM, disregarding the few crystals that precipitated out of solution). The cells were then cooled on ice for an additional ten minutes. The 0.5 ml quantity of cells were then placed into open-topped, flat sided cuvettes which were lined with two 0.025 mm thick aluminum foil electrodes (50 x 9 mm) mounted by epoxy glue on opposite sides of the cuvette. These cuvettes had been previously sterilized with 70 % ethanol and sterile PBS washes. The two groups to be electroporated received a single 0.5 second electrical pulse from an ISCO 494 power supply, which was preset to deliver 0.9 mA at a voltage of 2,000 volts. Current and wattage dials were positioned to a value of 5 (on a scale of 100) in order to obtain the correct electroporation parameters. The two tubes which were not to be electroporated were treated identically, except the power was not applied to their electrodes. Following treatment, the cell suspension was removed from the cuvettes and diluted with ice cold medium (RPMI + 10% fetal horse serum). The cells then received 2.5 ml of room temperature medium. Following a ten minute room temperature incubation, the volume of each of the four samples was brought up to 10 ml by the addition of 37 C fresh medium; they were then tranferred to tissue culture flasks, and left to incubate at 37 C for 1.5 hours. These incubations were to allow the cells to recover from the electroporation. The four tubes were each washed eight times with fresh room temperature medium. Washing consisted of adding 10 ml of media, centrifuging, and resuspending the cell-containing pellet. After this prepartion, cells were resuspended to achieve a concentration of 4×10^5 cells per ml. Smears of each of the four samples were made; 1 ml of each group was placed into fresh medium to determine viability by measuring ability to grow over a 72 hour period; the remainder of the cells were used in the deoxyuridine tritium release assay.

RESULTS

A: Characterization of PKC-1 and PKC-2 as potential novel anti-folates

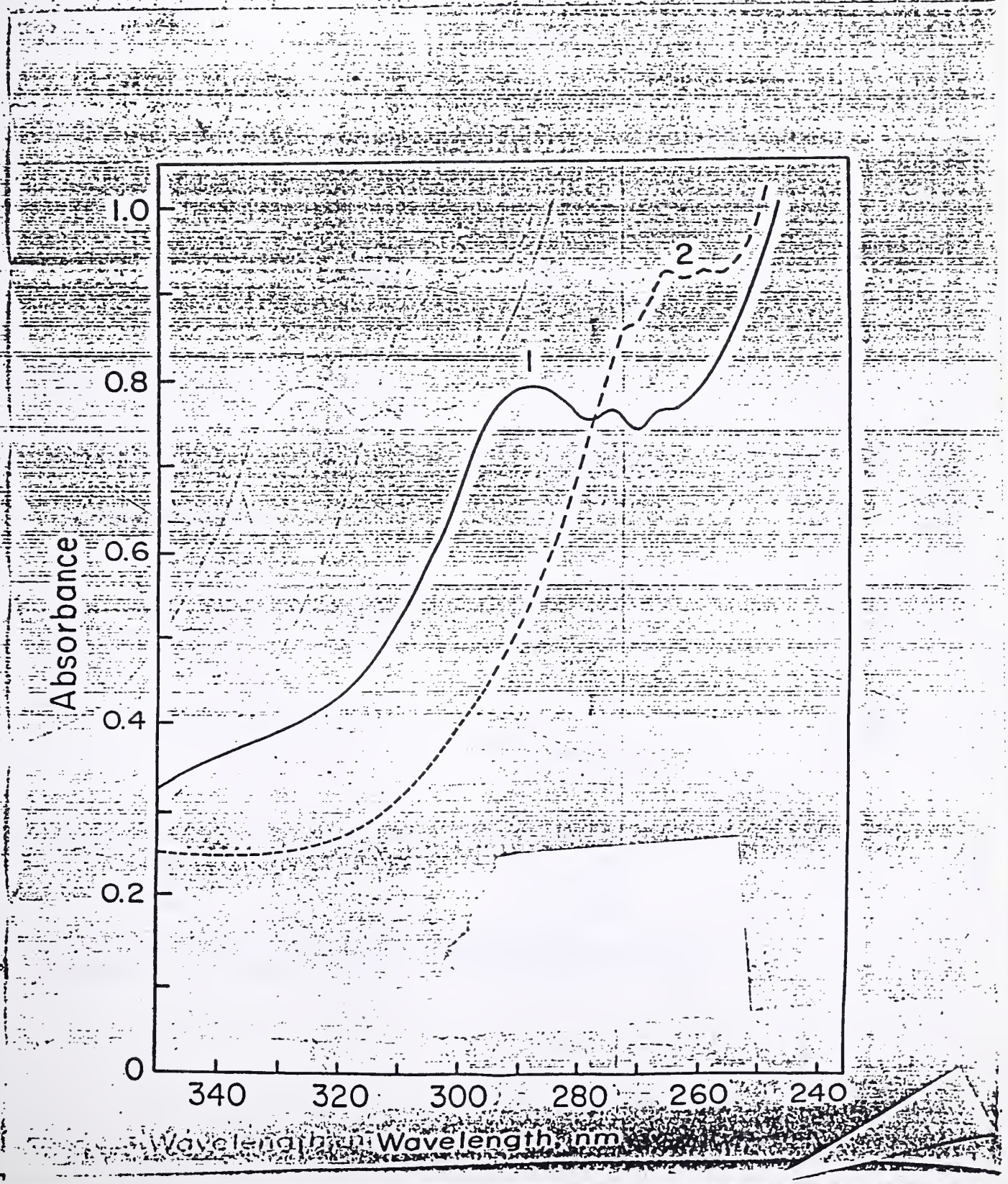
1. Ultraviolet and visible spectra:

Figure 8 demonstrates the U.V. and Visible spectra of PKC-1, 1-(2,4-diamino-6-pteridinyl)-2-3,4,5-trimethoxyphenyl(ethylene) and its 7,8-dihydro form, PKC-2, 1-(2,4-diamino - pteridinyl)-2-3,4,5-trimethoxyphenyl (ethane). PKC-1 demonstrates maximal absorbance at approximately 292 nm with secondary peaks at 274 nm (solid line). Similarly, the 7,8-dihydro analog is devoid of the 292 nm peak but demonstrates a unique profile, with maximal absorption at 287 nm and 274 nm (dotted line). The data represented are shown for both of these compounds dissolved in phosphate buffer (0.05 M) and adjusted to a pH of 7.0. When dissolved in HPLC grade methanol, 0.01 N HCl, and 0.1 N NaOH identical spectra were obtained. The spectra remained identical 24 hours after the solutions were prepared, as well as one week afterwards, indicating that the compounds are stable in solution (DMSO) maintained at 4 C.

2. Flourescence spectra:

Figure 9 illustrates the flourescence characteristics of PKC-1. An 8.6 x 10^{-4}M solution of PKC-1 was prepared by dissolution in DMSO. The Farrand flourescence spectrometer was set to a range of 1, with 5 nm slits. The left-most panel of figure 9 demonstrates that this compound is maximally excited at 400 nm; the right-most panel discloses that, when excited at 400 nm, maximal emmission occurs at 465 nm.

Figure 8.
Ultraviolet and visible spectra of PKC-1 ('1') and PKC-2 ('2')



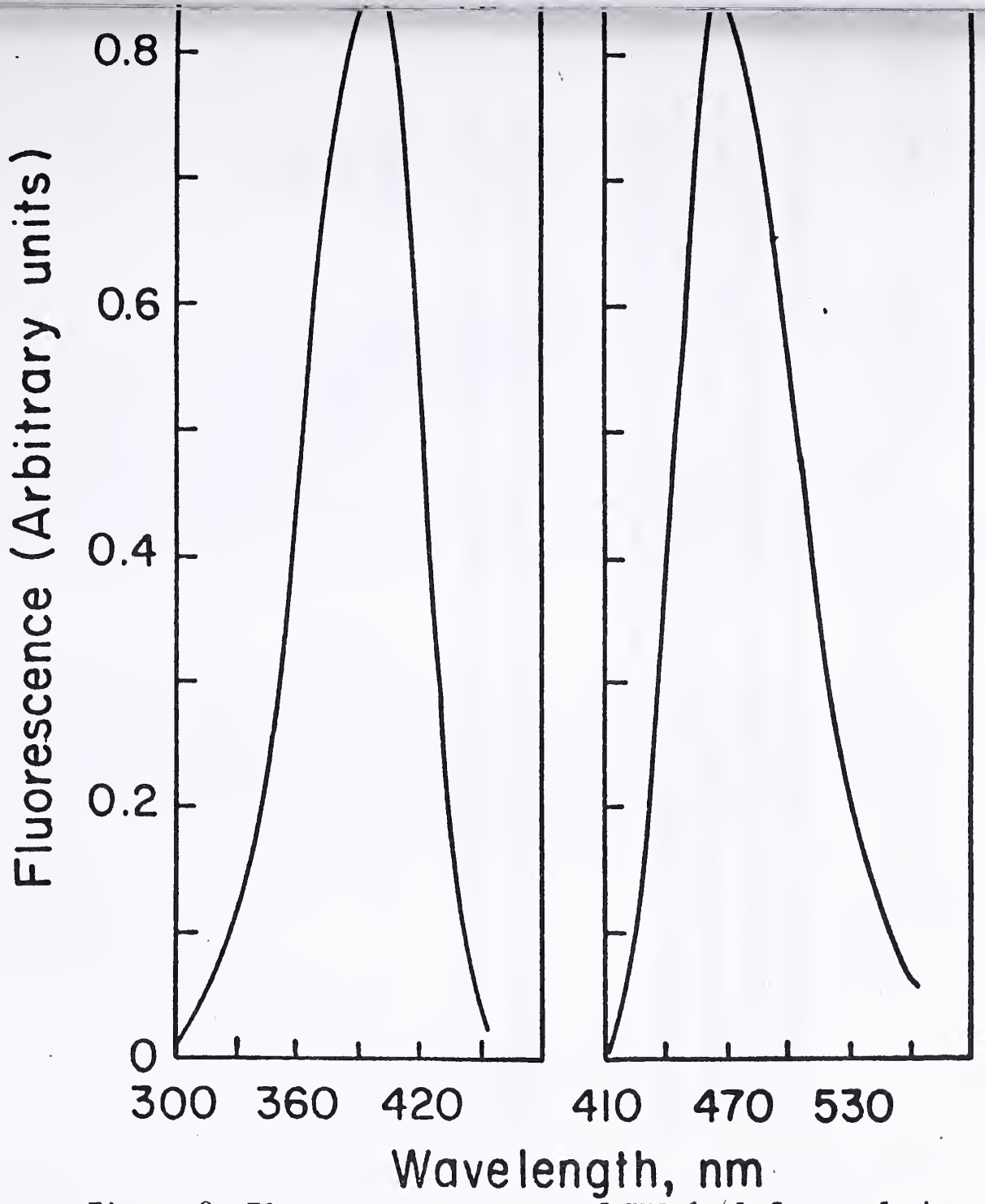


Figure 9. Flourescence spectra of PKC-1. Left panel shows excitation scan; right panel shows emmission scan.

3. DHFR Inhibition *in vitro:*

Both PKC-1 and PKC-2 were initially tested against a DHFR derived from an L5178Y isolate and it was predicted that the 7,8-dihydro compound (PKC-2) would be an excellent inhibitor of this enzyme. IC_{50} values were computed as previously described in the materials and methods section. The following results were obtained:

Compound	IC_{50}
PKC-1	$1x\ 10^{-4}$ M
PKC-2	$5x\ 10^{-5}$ M
MTX	0.9×10^{-9}M (Shown for purposes of comparison)

The compounds were then tested against DHFR derived from Lactobacillus caseii. The rationale for this test was that these compounds, which were predicted on the basis of their structures to have inhibitory activity against the mammalian form of DHFR, but demonstrated only minimal activity, may have inhibitory properties against another form of this enzyme. An example of such a compound is trimethoprim, an antibiotic, which selectively inhibits bacterial DHFR but has no activity against the mammalian form.

As demonstrated here, a spectrum of inhibition similar to the mammalian enzyme form was noted when the L. caseii form of the enzyme was employed, with both compounds having IC_{50} values in the range of 1×10^{-4}M.

Compound	IC_{50}
PKC-1	1×10^{-4} M
PKC-2	1×10^{-4} M
MTX	0.5×10^{-8}M

4. Cytotoxic effects of PKC-1 and PKC-2:

As described in the materials and methods section, these drugs were tested against cells growing in culture. As illustrated below, both of these compounds were not effective inhibitors of cell growth against either the CCRF-CEM, a liquid tumor, or against a squamous cell, solid tumor cell line(SC):

Compound	Cell line	Calculated ED_{50}
PKC-1	CEM	3×10^{-4} M
PKC-1	SC	5×10^{-4} M
PKC-2	CEM	5×10^{-5} M
PKC-2	SC	1×10^{-5} M
MTX	SC and CEM	1×10^{-9} M

B. Characterization of flourodeoxyuridine-MmC:

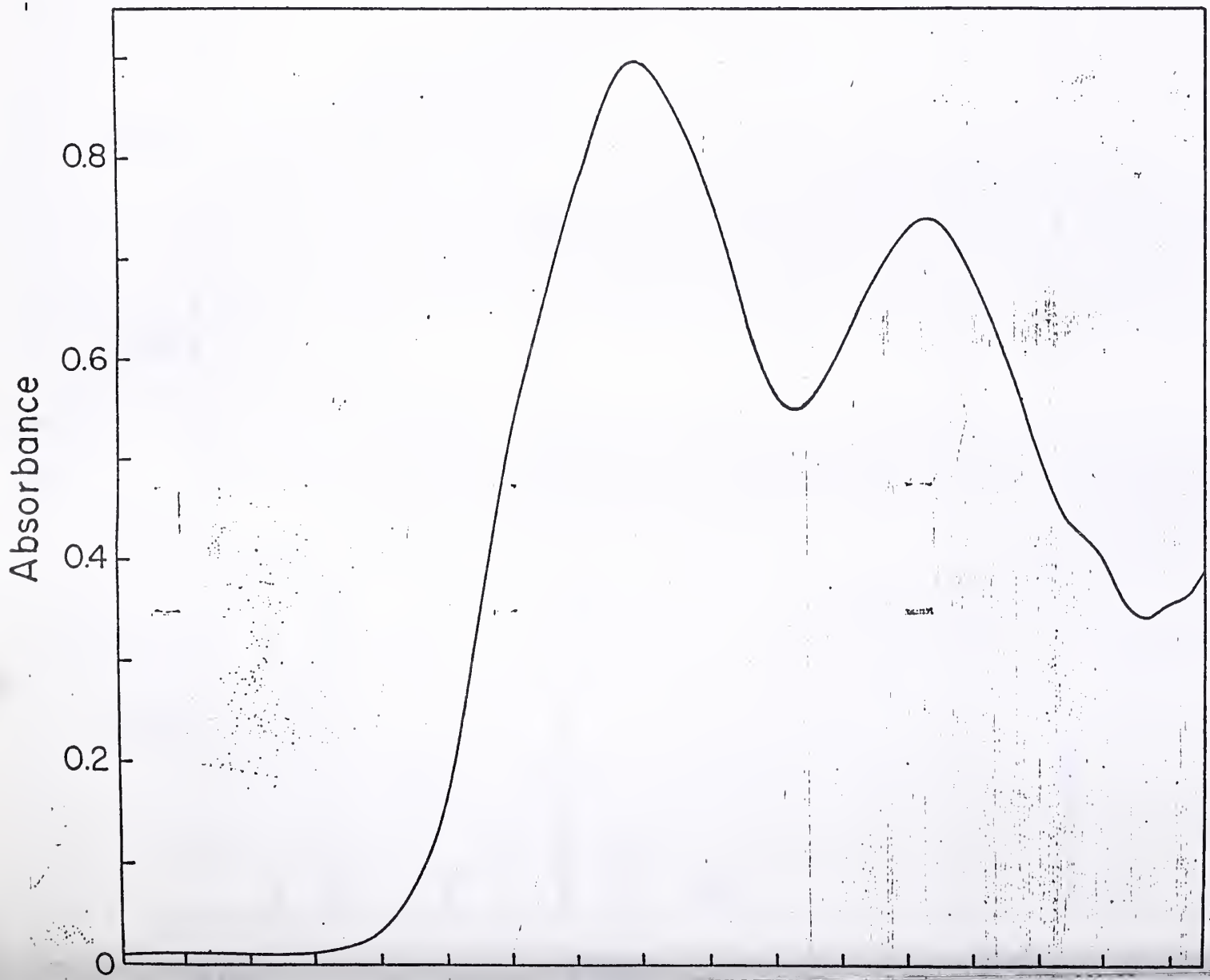
1. Ultraviolet and Visible Spectra; mass spectroscopy:

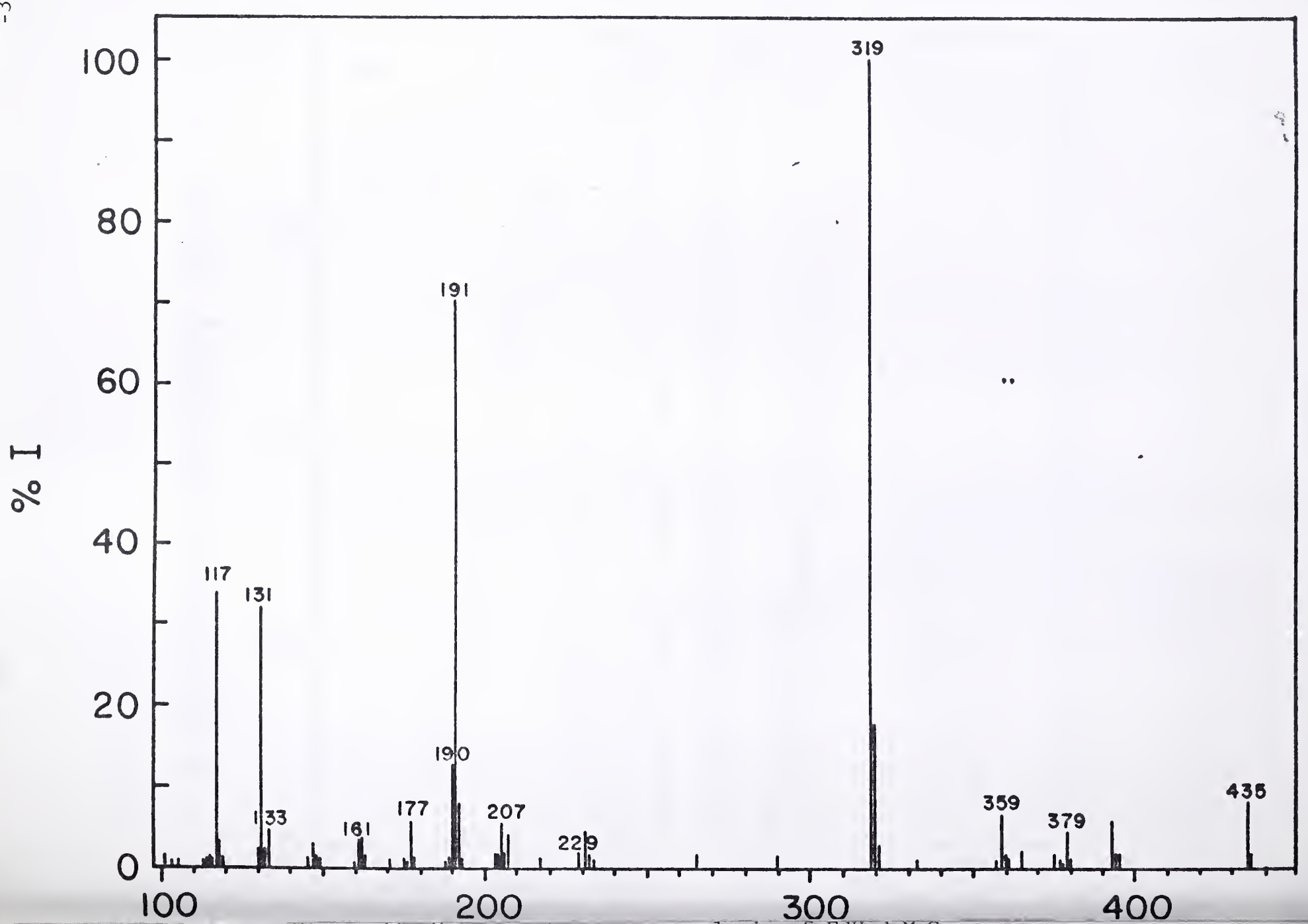
Figure 10 demonstrates the spectra of FdUrd-MmC, with absorption peaks at 320 and 276 nm. Twenty-five micrograms of FdUrd-MmC were dissolved in 1.0 ml HPLC grade methanol. Similarly, figure 11 discloses the mass spectroscopy data on this compound. UV and visible spectra were repeated twenty-four hours later, with no changes noted. It therefore appears that FdUrd-MmC is stable, at least over twenty-four hours, when dissolved in methanol. In later experiments, it was appreciated that FdUrd-MmC will precipitate out of solution when stored overnight at 4 C at a concentration greater than or equal to 20 mM.

2. Flourescence spectra:

Figure 12 shows the flourescence spectra of FdUrd-MmC. A 2 mM stock solution was prepared by dissolving this compound in HPLC grade methanol. 50 ul of this stock solution of FdUrd-MmC were further diluted into 2.0 ml of methanol. The Farrand Optical flourometer was set for a range of 1.0. The compound maximally flouresced at an excitation frequency of 335 nm (figure 12.). An emission scan (figure 12, right panel) at an excitation frequency of 335 nm disclosed maximum emmission at 400 nm. Identical results were obtained when the stock solution was added to phosphate buffered saline (PBS). Furthermore, these flourescence characteristics were identical in another batch of FdUrd-MmC prepared at a later time and persisted for at least twenty-four hours. It appears that FdUrd-MmC is a truly flourescent analog, with easily identified, discrete, excitation and emmission peaks.

Figure 10. UV and visible spectra of FdUrd-MmC dissolved in methanol.

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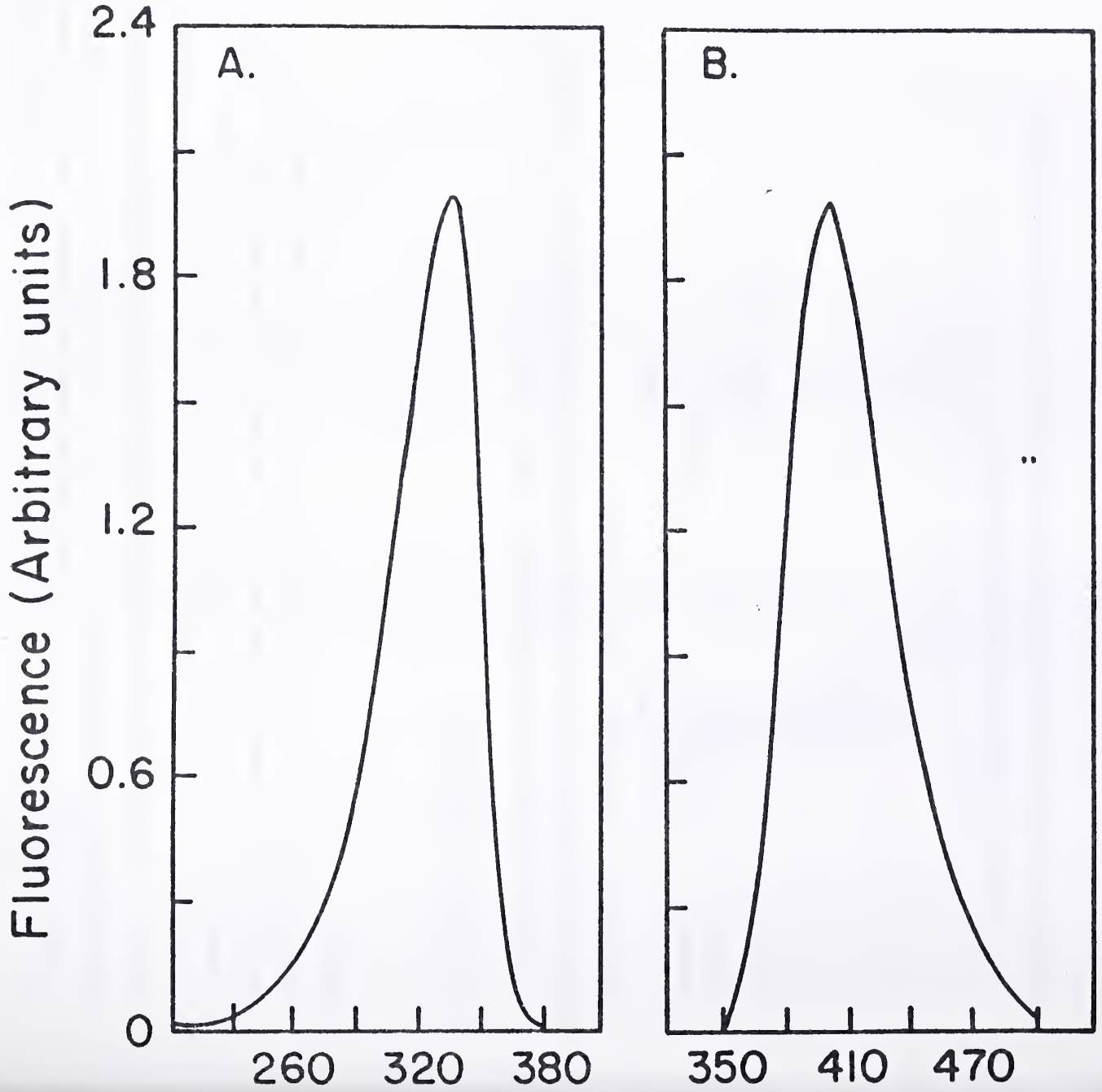

Figure 12. Flourescence spectra of FdUrd-MmC.

3. FdUrd-MmC entry into cells:

Once the flourescence characteristics of this uridine analog were defined, it was necessary to determine if it was able to passively enter cells. CEM-CCRF cells were placed into fresh media containing 3 uM of FdUrd-MmC. After one hour, cells were transferred onto microscope slides (as a smear), and examined under a flourescence microscope. If the cells were able to take up and concentrate this analog they ought to have demonstrated flourescence. None of the cells, however, were flourescent. The same procedure was repeated, and the cells remained for twenty-four hours in a 3 uM solution of FdUrD-MmC. Upon examination under the flourescence microscope several broken cellular fragments vividly flouresced. None of the intact cells demonstrated flourescence. On the basis of these experiments, it was concluded that FdUrd-MmC is unable to passively enter cells and diffuse through membranes.

4. Cytotoxic effects of FdUrd-MmC:

Despite the previous finding that FdUrd-MmC is unable to passively enter cells, the ability of this novel compound to kill cells was assessed. As described in the materials and methods section, CEM-CCRF and SC cells were incubated with varying concentrations of this compound:

Concentration	Number of CEM-CCRF cells	Number of Squamous cells
0 (Control)	12.5 *	5.3 *
1 x 10^{-9} M	11.5	6.2
3 x 10^{-9} M	12.5	5.8
3 x 10^{-8} M	12.0	5.4
1 x 10^{-6} M	14.5	6.3
3 x 10^{-6} M	9.5	6.1

* x 10^{5} cells per ml. Cells were incubated for forty-eight hours.

Clearly, at a concentration of 3 uM, FdUrd-MmC is not cytotoxic to either of the two cell lines tested.

5. Electroporation of FdUrd-MmC into CEM-CCRF cells:

As FdUrd-MmC does not passively enter cells, electroporation was used to introduce this compound. Initially, CEM cells were electroporated in the prescence of 100 uM of FdUrd-MmC and then examined under the flouresence microscope. It was noted that approximately ten percent of the cells flouresced, effectively demonstrating that the compound was introduced via electroporation.

In a subsequent experiment, CEM cells were electroporated in the prescence of 1 mM FdUrd-MmC. Four groups were designated: 1) FdUrd-MmC with electroporation, 2) FdUrd-MmC without electroporation, 3) electroporation without FdUrd-MmC and 4)no electroporation or FdUrd-MmC. Smears were made of each group and examined under the flouresence microscope. Cell viability was determined for each group by measuring absolute number of cells at 0, 24, 48 and 72 hours after suspension in fresh media. Finally, thymidylate synthetase activity and/or thymidine kinase activity as measured by ^{3}H-deoxyuridine incorporation was assessed. Prior to this experiment, FdUrd-MmC was dissolved in 1.0 ml of 50 % HPLC grade methanol and 50 % PBS to a final concentration of 20 mM. This solution was stored overnight at 4 C. The morning of the electroporation it was noted that some crystals had precipitated out of solution. The stock solution was warmed, which led to more dissolution of crystals. Some of the compound, unfortunately, did not dissolve and the final concentration of FdUrd-MmC was not re-determined. Nonetheless, the solution was employed in the experiments.

a. Appearance of smears under the flouresence microscope:

Smears from all four groups were prepared in duplicate and examined under the flouresence microscope. Despite extremely vigorous washings (eight washings in fresh media) crystalline material was noted in all smear preparations except the fourth group which had no compound. The material appeared as a snowflake pattern interspersed on a background of intact CEM cells. Strangely, neither the cells nor the crystals flouresced. If the crystalline material, which almost certainly represents FdUrd-MmC or some derivative thereof, did not flouresce, the stability of this compound must be questioned. Conceivably, the process of precipitating out of solution and then being re-dissolved led to the displacement of the MmC group from the FdUrd. However, 1) the MmC itself ought to have flouresced and 2) previous flouresence scans and UV/ visible spectra demonstrated that re-suspension of FdUrd-MmC did not alter the characteristics of this compound.

b. Cell viability:

After electroporation and incorporation of FdUrd-MmC, each of the four groups were tested for cellular viability. Specifically, 0.1 ml of each group were added to 10 ml of fresh media. Absolute cell count was determined after re-suspension in this fresh media; subsequent cell counts were taken at 24, 48 and 72 hours later. Each group was tested in duplicate and absolute cell counts are presented as an average of these duplicates:

Time(hrs)	F.M.* + E.P. **	F.M. no E.P.	No F.M. + E.P.	No F.M. no E.P.@@
0	4.9 @	8.6	4.3	2.5
24	8.0	13.5	8.3	4.9
48	18.0	22.0	19.0	12.0
72	40.0	45.0	42.0	35.0

*F.M. = FdUrd-MmC **E.P. = Electroporation @ Cell counts are all x 10^4 cells/ml

@@The control group initially had 2.5 x 10^4 cells per ml as there was a shortage in the number of cells available after processing for the experimental conditions. Therefore, the control group received only half the volume of processed cells. All other groups received identical volumes of cells (0.5 ml).

It is interesting to note that both of the electroporated groups had half as many cells as the non-electroporated group (in the prescence of FdUrd-MmC). This may be explained as destruction of cells from the electroporation treatment itself.

c. Inhibition of DNA synthesis:

The conversion of uridine monophosphate to thymidine monophosphate, catalyzed by the enzyme thymidylate synthetase, is crucial in the manufacture of DNA. The conversion of uridine monophosphate to thymidine monophosphate was measured utilizing a tritium release assay, as described in the materials and methods section. After the CEM-CCRF cells were electroporated in the prescence and abscence of FdUrd-MmC, inhibition of DNA synthesis was estimated using a ^{3}H-deoxyuridine release assay. More specifically, this assay demonstrates either inhibition of uridine phosphorylation or inhibition of thymidylate synthestase, both of which are necessary for DNA synthesis. The assay was performed at two, three, four and twenty-four hours after electroporation. As described earlier, four groups were tested: 1)FdUrd-MmC and electroporation (designated as 'D+E') ; 2)FdUrd-MmC without electroporation (designated as 'D') ; 3)No FdUrd-MmC but with electroporation (designated as 'E') and; 4)neither FdUrd-Mmc nor electroporation (designated as 'C'). Data for the four time points is presented as a per-cent of control (the last group), which is taken as 100 %. For example, if a certain treatment inhibits DNA synthesis by 75 % then this group would demonstrate a 25 % incorporation of uridine into DNA relative to the control (always taken as 100 %). If an inhibitor of DNA synthesis is degraded intracellularly or is eliminated from cells by any mechanism, a recovery in DNA synthesis capability should be observed over time. Thus, immediately after exposure to such a drug, there might be a 95 % inhibition of DNA synthesis, (represented as 5 % of control); similarly, after twenty-four hours the cells may recover and manifest no inhibition of DNA synthesis and incorporate uridine as effectively as the control group. Results are depicted in table 3.

Table 3:

Tritium release from ^{3}H-2'-deoxyuridine

Condition	Time	% DNA synthesis (relative to control)*
C	2 hrs	100.0
E	2 hrs	5.7
D	2 hrs	5.2
D+E	2 hrs	8.3
C	3 hrs	100.0
E	3 hrs	10.0
D	3 hrs	8.9
D+E	3 hrs	13.0
C	4 hrs	100.0
E	4 hrs	16.9
D	4 hrs	15.3
D+E	4 hrs	7.1
C	24 hrs	100.0
E	24 hrs	34.1
D	24 hrs	33.7
D+E	24 hrs	43.8

* Data represent one data point and are expressed as % of control.

DNA synthesis capability at different time points of CEM-CCRF cells after various treatments. C= control (no FdUrd-MmC, no electroporation); E= electroporation in the abscence of FdUrd-MmC; D= FdUrd-MmC in media, but no electroporation; D+E= FdUrd-MmC and electroporation. Results are expressed as a percentage of control, which was taken as 100 % DNA synthesis capability. Results represent scintillation counts of the released tritium, as previously described.

DISCUSSION

A. PKC-1 and PKC-2 as novel anti-folates:

PKC-1 and PKC-2 are both novel 2,4-diamino pteridines. They exhibit intrinsic flourescence, with discrete spectra. Flourescent enzyme inhibitors would be very useful in elucidating intracellular properties. For example, cells which have amplified the dihydrofolate reductase gene would themselves become vividly flourescent if an intrinsically flourescent molecule bound tightly to DHFR. The additional copies of DHFR would allow many molecules of the flourescent compound to bind, thereby making the cells visible. This would potentially allow rapid determination, without the use of radioactive probes, on the state of a patient's cells. It would be very useful to be able to determine if the cells have an increased level of a target enzyme, and thereby modify the patient's chemotherapeutic regimine. If a patient had cells that did demonstrate abnormally high levels of an enzyme, such as DHFR, then 1) increasing the dose of an anti-folate or 2) switching to a different class of antineoplastic drug would be warranted. This would reduce the patients exposure to toxic agents which, given that the target enzyme is greatly amplified, would probably not be efficacious. PKC-1 and PKC-2, structurally similar to the 2,4 diamino- pteridine trimetrexate, were designed to inhibit DHFR. PKC-2, with its fully saturated carbon-carbon bond, was predicted to potently inhibit this enzyme. The carbon-carbon bond in this compound was thought to allow necessary freedom of movement thereby permitting this molecule to avidly bind to DHFR. The data in this paper show that neither PKC-1 nor PKC-2 inhibited either a mammalian or a bacterial form, derived from Lactobacillus caseii, of DHFR. Furthermore, neither of these two compounds had appreciable cytotoxic activity against either a leukemic cell line (CEM) or a solid tumor (S.C.). The finding that these compounds are not cytotoxic is not surprising as these drugs demonstrate no significant activity against DHFR.

Of note was the fact that neither of these compounds were DHFR inhibitors, especially given that many 2,4-diamino pteridines are potent antagonists. This may be explained in several ways. First, it is possible that these compounds are unstable in aqueous media. Second, these drugs may have been rapidly metabolized intracellulary into an inactive form. Third, owing to the lability of the methoxy groups, it is possible that the overall structure of these molecules became disrupted upon loss of of a single methoxy group. To test these hypotheses, confirmation of the structure of these compounds is required. Specifically, nuclear magnetic resonance (NMR) spectroscopy of these compounds, both before and after dissolution into a cellular media, would facilitate an accurate identification of their structures. If these compounds do prove to be unstable, halogenation at one of the methoxy positions may act to stabilize the molecule. As shown in table one, many halogenated 2,4-diamino pteridines are potent DHFR inhibitors. A dimethoxy, halogenated derivative of PKC-2 may be stable, flourescent and a potent DHFR inhibitor. It is therefore suggested that NMR analysis, as well as testing halogenated compounds, be further studied in order to ultimately derive a flourescent DHFR inhibitor.

B. Characterization of FdUrd-MmC as a flourescent molecular probe:

FdUrd-MmC is a flourescent analog of FdUrD and is a potential molecular probe for the study of the physiology of neoplastic cells. A flourescent compound which becomes incorporated into DNA should permit direct visualization of a number of cellular processes. FdUrd-MmC was studied as a prototype compound to attempt to visualize pathways of uridine metabolism in a leukemia cell line (CEM).

On the basis of its flourescence characteristics, it was first determined whether or not FdUrd-MmC can enter cells. This compound was unable to diffuse through membranes and only demonstrated flourescence on broken cellular fragments. Nucleosides enter cells via an active transport carrier mechanism. At high concentrations nucleosides can enter cells by passive diffusion. FdUrd-MmC was not transported by the active carrier or by passive diffusion, as evidenced by the lack of intracellular flourescence. That this compound did not enter cells either passively by diffusion or actively via the nucleoside specific uptake mechanism may be attributed to its inability to be actively transported because of its large size and its structure. Conformationally, one domain of this large molecule is hydrophilic and an opposing domain is hydrophobic, thereby limiting its capacity to easily diffuse through membranes. This may account for why membrane fragments vividly flouresced, but whole cells did not. Furthermore, since this compound was unable to enter cells, it was not surprising that it did not demonstrate any cytotoxic effects, even at concentrations in the micromolar range. If FdUrd-MmC could be introduced into cells might it, or a metabolite of this agent, be capable of inhibiting DNA synthesis? FdUrd-MmC was experimentally introduced into CEM cells.

Electroporation is a new technique which allows the incorporation of impermeable compounds into cells. This procedure consists of a brief electrical discharge which is thought to transiently disrupt normal membrane function. FdUrd has recently been introduced into cells (Jastreboff, et al, in press) and has been shown to become incorporated into DNA. FdUrd-MmC was tested using similar techniques. In one experiment, FdUrd-Mmc was successfully electroporated into CEM cells with approximately ten per-cent of the cells demonstrating flourescence. When electroporation of FdUrd-MmC was repeated using a concentration of 1 mM, the compound precipitated out of solution and no longer demonstrated flourescence. FdUrd-MmC is soluble in methanol but insoluble in H_2O or PBS. It is therefore difficult to obtain intracellular concentrations in the millimolar range without the compound rapidly precipitating out of solution. Adding FdUrd-MmC dissolved in pure methanol would be toxic to cells. Future experiments with this compound will require a non-toxic solvent. A suitable solvent to employ is dimethyl sulfoxide (DMSO). Without a good, non-toxic solvent, it is difficult to generate and interpret data when studying FdUrd-MmC.

Electroporating a compound that is not fully solubilized has several major problems. Most significantly, non-dissolved particulate matter acts ballistically, disrupting the integrity of the cellular membrane. Despite the previously stated problems, a limited interpretation of data in this paper is possible. Incorporation of FdUrd-MmC into cells via electroporation did not result in cytotoxicity. However, as mentioned previously, smears of cells treated with FdUrd-MmC demonstrated two unexpected findings. First, the compound precipitated out of solution and appeared as a snow-flake pattern. Second, the precipitate itself did not flouresce. It is therefore unclear what happened to this compound once it was dissolved into the cells. The loss of flourescence is highly suggestive of the compound being structurally unstable under the conditions of the experiment. DNA synthesis, as measured by the ^{3}H-deoxyuridine tritium release assay, was decreased by both electroporation alone and by FdUrd-MmC alone. The combination of FdUrd-MmC and electroporation also inhibited DNA synthesis. That FdUrd-MmC inhibited DNA synthesis is preliminary evidence that this compound or one of its metabolites is an active uridine antagonist. Within twenty-four hours, the cells that had been treated with electroporation, FdUrd-MmC, and the two together, manifested a partial recovery of approximately forty per-cent in their ability to synthesize DNA. It is interesting that electroporation alone would lead to a ninety-five per cent reduction in DNA synthesis; mechanisms for this finding remain obscure. Other investigators (Jastreboff, et al, in press) did not obtain this effect from electroporation alone, and it may be artifactual in the experiments reported herein. Further experiments will disclose whether or not electroporation alone inhibits DNA synthesis. Nonetheless, these preliminary experiments should be repeated, using an appropriate solvent system such as DMSO. Furthermore, FdUrd should be employed as a control so as to determine if FdUrd-MmC behaves identically to the non-flourescent form of the uridine analog.

A flourescent compound such as FdUrd-MmC may be useful in characterizing the effectiveness of electroporation. A flourescent compound, normally impermeable to cells, can be electroporated. The flourescence of the cells would indicate the number of molecules of the flourescent compound that was effectively electroporated into the cells. The parameters which effect electroporation may then be quantized by using a technique such as flourescence activated cell sorting(FACS). Thus, the parameters for electroporation would be able to be optimized.

The electroporation of a flourescent nucleotide analog may ultimately aid in the elucidation of many aspects of cellular functioning. Methodologic problems, such as solubility, still need to be resolved, but this new technique remains a potentially powerful research tool. Flourescent compounds that selectively label enzymes, and other cellular elements, may enable more direct visualization of intracellular processes. The PKC compounds and FdUrd-MmC represent a first step in developing a molecular technology which may one day allow a visual demonstration of cellular events.

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